A 6-weeks supplementation with grape pomace to subjects at cardiometabolic risk ameliorates insulin sensitivity, without affecting other metabolic syndrome markers

Daniel Martínez-Maqueda[a,b], Belén Zapatera[a], Angélica Gallego-Narbón[a], M. Pilar Vaquero[a], Fulgencio Saura-Calixto[a], Jara Pérez-Jiménez[a*]

[a] Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), José Antonio Novais 10, 28040, Madrid, Spain.

[b] Current affiliation: Madrid Institute for Rural, Agricultural and Food Research and Development (IMIDRA), A-2 Km. 38.2, 28805 Alcalá de Henares (Madrid), Spain

[*] Corresponding author: Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), José Antonio Novais 10, 28040, Madrid, Spain. Tel.: (+34) 91 549 23 00. E-mail: jara.perez@ictan.csic.es

Abstract

Grape polyphenols have shown a promising role in the modulation of metabolic syndrome (MetS), mostly in animal models. However, clinical studies are scarce and they usually only consider a fraction of polyphenols, ignoring the non-extractable polyphenols (high molecular weight compounds or associated with macromolecules such as dietary fibre). This study aimed to evaluate the effect of grape pomace, rich in both extractable and non-extractable polyphenols, on markers of MetS. Fifty subjects (22 women) aged 20-65 with at least two MetS factors were randomly assigned to the product (daily dose of 8 g of dried grape pomace) or to the control group in a 6 weeks crossover design with a 4 weeks wash-out. Samples were collected at the beginning and at the end of both periods; half of the participants were subjected to an Oral Glucose Tolerance Test at the beginning and the end of the supplementation period. Grape pomace supplementation significantly improved fasting insulineamia ($p<0.01$), without affecting other cardiometabolic risk parameters. A tendency towards an improvement in postprandial insulineamia was observed, particularly in those subjects with higher fasting insulin levels. Therefore, supplementation with grape pomace may be a strategy for improving insulin sensitivity in subjects at high cardiometabolic risk.

Keywords: Metabolic syndrome; insulin sensitivity; polyphenols; grape pomace.

Abbreviations

AUC: area under the curve.

BMI: body mass index.

DF: dietary fiber.

HDL: high-density lipoprotein.

HOMA-IR: homeostatic model assessment-insulin resistance.

LDL: low-density lipoprotein.

MetS: metabolic syndrome.

OGTT: oral glucose tolerance test.

INTRODUCTION

Metabolic Syndrome (MetS) is a cardiometabolic situation that comprises the combination of several risk factors (abdominal obesity, hyperglycaemia, hypertension and dyslipidemia, among others) associated with a sedentary lifestyle. Although it is not a disease *per se*, MetS may lead to the onset of type 2 diabetes or different cardiovascular pathologies [1]. The main underlying mechanism in MetS is insulin resistance, combined with increased oxidative stress and chronic low-level inflammation [2, 3]. It has been estimated that the prevalence of MetS is 20-30% of adults in developed countries[4]; therefore, finding new strategies for tackling MetS is a relevant challenge.

Within this context, there is particular interest in the identification of nutritional strategies that, as previous step to drug approach, may ameliorate the disturbances present in MetS. Polyphenols, a diverse family of plant secondary metabolites widely spread in plant foods, are promising agents in that sense. In particular, there is cumulative evidence through *in vitro* and preclinical studies that polyphenols may be able to regulate some of the physiological processes altered in MetS by different mechanisms of action [5-11]: modification of postprandial glycaemia due to interactions with intestinal glucosidases and glucose transporters; improvement of insulin and adiponectin signalling pathways; reduction of oxidative stress and inflammation; hypotensive effect; hypolipidemic effect by repressing intestinal lipid absorption, triglyceride content in skeletal muscle and chylomicron/VLDL secretion.

Many studies on the role of polyphenols in the modulation of MetS have been performed with berries due to their high polyphenol content, mostly proanthocyanidins and anthocyanins. Thus, beneficial effects on inflammation markers, lipid profile or insulin resistance have been reported after the supplementation with wild blueberries, ellagitannin-

rich berries or ginseng berry, respectively [12-14]. More specifically, grape derived products such as grape seed extracts have shown beneficial effects in the same sense [7]. However, these extracts only contain a fraction of polyphenols -the extractable polyphenols- while their derived residues present a high amount of the so-called non-extractable polyphenols or macromolecular antioxidants. They are high molecular weight compounds or compounds associated with macromolecules such as dietary fibre, with promising health-related properties[15]. Grape pomace is a material commonly discarded during the wine-production process with a high content in both extractable and non-extractable polyphenols[16], being therefore a very interesting product to be tested for the effect of its polyphenols as a whole in the modulation of MetS as well as an important opportunity for the environmental sustainability. In addition, although a significant number of animal studies have explored the effect of grape polyphenols on MetS markers, clinical trials on the subject have been much limited and with contradictory results [17].

Therefore, the aim of this study was to evaluate the capacity of a long term supplementation at nutritional doses of grape pomace (containing both extractable and non-extractable polyphenols) to modulate metabolic disturbances present in subjects at cardiometabolic risk related to MetS.

EXPERIMENTAL

Dietary supplement

This study was performed with a nutritional supplement constituted by dried and milled grape (*Vitis vinifera* L., cv Tempranillo) pomace. It was collected fresh -at the moment of wine devating- from Roquesan Wineries (Quemada, Burgos, Spain), being later transported at -20ºC, freeze-dried, ground to a particle size of 0.5 mm and sealed in monodoses (8 g). These monodoses were stored at -20ºC until the beginning of the study, three months later.

We had previously verified that freeze-dried grape pomace, under freezing conditions, kept a constant polyphenols composition; moreover, in case some degradation took place, this would affect to the minor extractable fraction of polyphenols and not to the major non-extractable polyphenols. Prior to the intervention study, standard microbiological (*E.coli* β-glucuronidase, *L. monocytogenes*, *Salmonella* spp., viable aerobes at 30ºC and fungi/yeast) and metal (Pb, Cd, Ni, Zn, As and Sb) analyses were carried out to guarantee the safety of the product.

Dried grape pomace was characterized by a high polyphenol content (29.63 %), particularly non-extractable polyphenols or macromolecular antioxidants (23.44 %), as well as a by a very high content in dietary fiber (68.23 %), mostly insoluble (65.65 %). More information on the composition of the product, as well as the methods used for its evaluation, are provided as Electronic Supplementary Information (Table S1). Besides, the detailed polyphenol composition of this product -combined with pomegranate pomace- was reported elsewhere[16]. It should be remarked that, since this dietary supplement is not an extract, but the whole grape pomace matter only subjected to drying and milling, other studies have described the composition of such material [18] .

Subjects and study design

This study was a randomized cross-over controlled clinical trial approved by the Ethics Subcommittee of the Spanish National Research Council (CSIC), Madrid, Spain (2016/12/13) and the Ethics Committee for Clinical Research of the University Hospital Puerta de Hierro-Majadahonda, Majadahonda, Spain (2016/12/02). It was registered in the Clinical Trials database with the identifier NCT03076463. The study was conducted between December 2016 and July 2017. All the subjects signed an informed consent form agreeing to participate in the study.

144 Subjects were recruited via poster advertisements and mailing sent to academic, social,
145 sanitary, and research institutions, in addition to personal contacts in Madrid, Spain.
146 Inclusion criteria for the study were to be aged 18-70 years, apparently healthy and to fulfill
147 at least two of the following requirements, based on official criteria for the diagnose of
148 MetS [1]: Body Mass Index > 25 kg/m^2; fasting glucose ≥ 100 mg/dL; HDL-cholesterol ≤ 50
149 mg/dL in women and 40 mg/dL in men; triglycerides ≥ 150 mg/dL; systolic pressure ≥ 130
150 mmHg or diastolic pressure ≥ 85 mmHg. Exclusion criteria were: diagnose or medication
151 for cardiometabolic pathologies; being pregnant or lactating; current or close participation
152 in any other dietary intervention study.
153 The study included two different 6 week periods, separated by a 4 weeks wash-out: a
154 supplementation period (daily supplementation with 8 g of product described above
155 suspended in water) and a control period (no appropriate placebo was found). Since it was a
156 cross-over design, subjects were randomly allocated to two groups, each one of them
157 starting by one of the periods. There was a difference of 10 weeks (6 of supplementation
158 and 4 of wash-out) between those subjects starting with the control period and those with
159 the supplementation period; this lag-time does not affect the polyphenol composition of the
160 product, as explained above. Volunteers were instructed to follow their habitual diet and
161 daily activities, as well as to store the monodoses of the product under freezing conditions.
162 Samples collection and measurements were performed at the beginning and at the end of
163 each one of these periods. Besides, at the beginning and at the end of the supplementation
164 period, half of the participants were subjected to a fasting OGTT (oral glucose tolerance
165 test) that consisted of ingesting 75 g of glucose in 200 mL of water; therefore, glucose
166 homeostasis parameters were evaluated only in these subjects. In order to limit the potential
167 effect of a high dietary polyphenol consumption close to the visits, 72 h prior to each visit

168 day, the subjects were required to refrain from consumption of polyphenol-rich foods such
169 as wine, coffee, tea, cocoa, whole bread, virgin oil olive, nuts, legumes and certain fruit and
170 vegetables such as berries or artichoke. A detailed list was provided to the volunteers.

171 **Sample size calculation**

172 The primary outcome variable for sample size calculation was the modification in HOMA-
173 IR (homeostatic model assessment for insulin resistance) index. In particular, power
174 calculations were based on a 30% reduction in HOMA-IR (or 10% increase in QUICKI,
175 quantitative insulin-sensitivity check index, based on the same parameters) following
176 previous nutritional clinical trials in subjects with impaired glucose tolerance supplemented
177 with polyphenol-rich materials [19]. A sample size of 40 was calculated as sufficient to detect
178 this change with 95% power and an alpha value of 0.05, using published variances of this
179 parameter [19]. This number was increased to 50 to ensure statistical power was sufficient
180 even if an important proportion of the subjects failed to complete the trial.

181 **Sampling and biochemical analysis**

182 Fasting blood samples were collected at the beginning and the end of each period between
183 8:00 and 10:00 h and after at least 10 h of fasting. During the OGTT study, blood was
184 collected at times 0, 30, 60 and 120 min. Serum and plasma were obtained after
185 centrifugation at 1000 *g* for 15 min and stored at -80 ºC. First morning urine was collected
186 and stored in aliquots at -80ºC.

187 The following parameters were measured by different automatic analyzers (Siemens
188 Healthineers, Tarrytown, NY, USA): ferritin by ADVIA Centaur XP; fibrinogen by
189 Sysmex C5-5100 Hemostasis System; serum total cholesterol, HDL cholesterol, LDL
190 cholesterol, triglycerides, iron, AspAT (aspartate aminotransferase), ALT (alanine
191 aminotransferase), and high-sensitive plasma C reactive protein by ADVIA Chemistry XPT

System; blood erythrocytes, hemoglobin, hematocrit, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), platelets, and MPV (mean platelet volume) by ADVIA 2120i Hematology system. The following analytes were determined by commercial ELISA kits according to manufacturer instructions: plasma insulin and leptin (Merck-Millipore, Burlington, MS, USA), and serum soluble transferrin receptor (DRG Instruments, Germany). Uric acid was measured in plasma and urine by a commercial kit (Spinreact, S.A., Sant Esteve de Bas, Spain). Determinations in urine were normalized by creatinine concentration, measured by a commercial kit (Cromatest, Linear Chemicals S.L., Montgat, Spain). Fasting and postprandial blood glucose was determined by applying the enzyme electrode method using a Free Style Optimum Neo blood glucose meter from Abbott (Chicago, IL, USA).

The areas under the curves (AUCs) for glucose and insulin were estimated using the trapezoidal function [20]. Insulin resistance was estimated with the HOMA-IR ([glucose (mg/dL)*insulin (μU/mL)]/405) and insulin sensitivity was estimated by the QUICKI index (1/[log insulin (μU/mL)+log glucose (mg/dL)]).

Blood pressure and anthropometric determinations

Blood pressure was measured at the beginning and the end of each period between 8:00 and 10:00 h in a quiet temperature-controlled room using an automated digital oscillometric device (Omron model M6 Comfort, Omron Corporation, Tokyo, Japan), and a mean of two readings was taken. Height, body weight and abdominal and hip perimeter were measured. Body composition was assessed by using a tetrapolar bioimpedance system (Tanita BC601, Arlington heights, IL, USA), including the following parameters (%): BMI, % body fat, % body water, muscle mass and bone mass.

Intestinal transit evaluation

At the end of the supplementation period, the subjects fulfilled a questionnaire with three questions (answered in a scale 0-10): “Did you perceive some modification in the number of bowel movements/amount excreted/stool consistency since you started to consume this product?”

Dietary assessment

Diet information was collected throughout the study via three 24 h dietary questionnaires (two for working days and one for weekend) applied before the study, and during the two periods (control/grape pomace). Data were processed for each 3 day-period by the DIAL System (Universidad Complutense de Madrid, Spain). Additionally, from the second visit the subject answered the question “Have you modified your physical activity since the last visit? If so, did you increase or decrease it?”.

Statistical analysis

Data were analyzed with the statistical SPSS IBM 24 package for Windows. First, outliers (>1.5 IQR) and extreme values (>3.0 IQR) were identified using box-and-whiskers plots, and extreme values were excluded from the results. Second, the possible effect of randomization (group assignment) was evaluated; for this, a t-Student test for independent variables was performed for basal values for each of the MetS factors between both groups (control and grape pomace), observing no statistical differences between groups. Therefore, data for all subjects when belonging to the control or to the grape pomace group, independently of the assignation order, were processed together. Subsequently, normal data distribution was evaluated based on kurtose coefficient (between -1 and +1) and, when it was not fulfilled, log transformation was performed (log + 1 when values were below 1). Finally, specific statistical analyses were applied for the different datasets: a) for diet evaluation (corresponding to three periods for each subject), analysis of variance (ANOVA)

240 followed by Tukey's test were applied; b) for glucose homeostasis measurements, t-student
241 test for paired samples was applied; c) for fasting measurements, the Mixed Linear Model
242 for repeated measures was applied and the effects of treatment (control or grape pomace),
243 time and time x treatment were studied. In all cases, differences with $P < 0.05$ were
244 considered significant.

245 **RESULTS**

246 **Subject characteristics**

247 The detailed CONSORT diagram of this clinical trial is shown in **Figure 1**. Briefly, from a
248 total of 234 subjects interested in the study, 48 started the first period. There was an
249 additional recruitment during the development of this period with three additional subjects
250 participating in only one arm of the study in order to increase the number of volunteers that
251 consumed the supplement; it was considered this was a better solution than having three
252 subjects prolonging the study until a different season. Therefore, considering drop-outs
253 during the study, data from a total of 49 subjects were analyzed. Overall, there was a high
254 adherence to the study, with one drop-out due to medical reasons (diagnose of severe
255 dislypidaemia) and two for sensory reasons.

256 The age of the subjects was 20-65, with a mean value of 42.6 (SE 1.6). Twenty-two
257 participants were females. The mean number of risk factors was 2.8 (SE 0.1), with a 63% of
258 the subjects fulfilling the official criterion for the diagnosis of MetS (at least three factors).
259 The distribution of the different risk factors among the subjects is shown in Table 1;
260 overweight/obesity, hypertension and hyperglycaemia were the most common risk factors
261 among them.

262 The comparative evaluation of the subjects' diet (before the study and during the two
263 periods) showed that there was no significant modification in any of its main parameters

(caloric content, macronutrient intake, selected micronutrient intake), as shown in Table S2. Additionally, although no detailed physical activity questionnaire was performed, 80% of the subjects did not modify their physical activity during the study based on a subjective perception (Table S3). Therefore, the potential effects observed during the supplementation period could be attributed to this supplementation.

Glucose homeostasis

Results for glucose and insulin levels during the OGTT are shown in Table 2. Fasting and postprandial glucose during the different sampling times were similar before and after the supplementation period. In contrast, basal insulin was significantly decreased ($P < 0.01$) after the supplementation with grape pomace. A non-significant tendency towards lower AUC 0-120 min after the supplementation (3,416 SEM 721 μU/mL x min), as compared with the value before supplementation (4,247 SEM 816 μU/mL x min), was observed. Moreover, when the subjects were stratified according to their 120 min insulin values (with a cut-off value of 50 μU/mL), it was observed that, after the treatment, a 93% of subjects with initial insulin values below 50 μU/mL kept within that range -as expected- but a 25% of the subjects with initial insulin values above 50 μU/mL moved below that value after the supplementation; these tendencies should be evaluated in further studies in order to confirm whether they actually reach significance. Insulin sensitivity and resistance indexes were also calculated, from fasting glucose and insulin values (**Table 3**). The supplementation with grape pomace caused a significant decrease ($P < 0.01$) in HOMA-IR, concomitant to a significant ($P < 0.05$) increase in QUICKI index; none of them were observed during the control period.

286 Leptin was also measured at fasting time and 120 min, as hormone closely related with
287 satiety and insulin sensitivity (**Figure** 2). Values at both sampling times as well as before
288 and after supplementation with grape pomace did not exhibit significant differences.
289 Finally, plasma uric acid was measured between fasting state and 120 minutes after glucose
290 overload, with no significant differences between either any of the sampling times or the
291 treatments (data not shown).
292 Glucose homeostasis, as indicated above, was only evaluated in half of the subjects;
293 therefore, these results only correspond to the pre-and post-supplementation samples,
294 without the strength provided by the cross-over design. Nevertheless, it should be stated
295 that, as shown below, there were no significant modifications during the control period in
296 all the parameters measured according to the cross-over design. It could be expected that
297 this was also the case for glucose homeostasis, although further studies should confirm this.
298 **Cardiometabolic risk factors**
299 **Table 4** shows the evolution in cardiometabolic risk factor during the study. No significant
300 time-treatment effect was observed. During both control and supplementation periods, a
301 significant ($P < 0.05$) decrease in fibrinogen was observed. Also, there was a slight
302 tendency towards the decrease of total cholesterol and LDL cholesterol, which was higher
303 in the supplementation period. Regarding uric acid, a tendency towards decrease was
304 observed in the supplementation period; nevertheless, uric acid values were higher in this
305 period than in the control period.
306 **Iron status markers**
307 Results for hemogram parameters and iron status are shown in **Table 5**. No significant
308 time-treatment effect was observed. During both control and supplementation period,
309 significant ($P < 0.05$) decreases in hematocrit and erythrocytes distribution index, as well as

310 increases in MCHC and MPV were observed. A tendency towards an increase in platelets
311 and decrease in ferritin was observed in both periods, although slightly higher in the
312 supplementation period.

313 **Anthropometric measurements**

314 **Table 6** indicates the results for anthropometric measurements. No significant differences
315 were observed either for general parameters (Body Mass Index, abdominal perimeter,
316 waist-to-height ratio, waist-to-hip ratio, metabolic age, visceral fat index), body
317 composition (fat, muscle bone mass, water) or abdominal composition (fat, muscle).

318 **Intestinal transit**

319 Intestinal transit was improved after grape pomace supplementation based on subjective
320 perception. Thus, a 57% of the subjects reported some increase in the total number of
321 depositions (with 26% rating between 8 and 10) and a 54% reported some increase in the
322 amount of feces excreted (with 21% rating between 8 and 10). Regarding consistency, 36%
323 of the subjects observed a tendency towards more liquid deposition although not implying a
324 drastic change in consistency (rates between 6-8); nevertheless, a 20% of the subjects
325 reported that grape pomace supplementation caused much more liquid stools (rates 9 and
326 10), while 16% observed an effect towards more solid depositions (rates below 5).

327 **DISCUSSION**

328 This study aimed to evaluate the potential of grape pomace to modulate parameters related
329 to MetS in subjects at high cardiometabolic risk. Despite the heterogeneity of the subjects
330 participating in the study –intrinsic to the definition of MetS-, it should be remarked that
331 they presented overweight, hypertension and hyperglycaemia as the most common cluster
332 of risk factors, i.e., the one previously reported as characteristic of Southern European
333 countries [4].

334 The specific approach of this study was to perform a supplementation with grape pomace,
335 rich in both extractable polyphenols and non-extractable polyphenols, commonly ignored in
336 studies of the topic[15]. The natural combination of both polyphenol classes in a single matrix
337 intrinsically associated with dietary fiber is a particular characteristic of grape pomace,
338 which therefore differs from the composition of other grape derived products [21].
339 Conversely, this limits the comparison of this clinical trial with others in the field of grape
340 and MetS -a topic on which a narrative review [22] and a systematic review [17] were recently
341 published- since clinical trials with grape pomace have been much scarce [23, 24]. Moreover,
342 the approach selected here means a complete use of the whole material generated during
343 wine processing, in contrast to others based on polyphenol-rich extracts from grape
344 pomace, leaving still a substantial amount of discarded material.
345 The supplementation -based on subjective perception with intrinsic limitations- seemed to
346 improve intestinal transit, what was expected from its insoluble dietary fibre content [25] and
347 previously reported for a grape pomace derived product [23]. The most remarkable effect was
348 that daily consumption of grape pomace in subjects at high cardiometabolic risk
349 significantly decreased fasting insulineamia. Moreover, the supplementation significantly
350 improved several insulin sensitivity indexes; in the case of HOMA-IR, the subjects moved
351 from original values corresponding to a situation of insulin resistance and MetS [26] to
352 normal HOMA-IR values. Interestingly, a tendency towards the regulation of postprandial
353 insulin sensitivity was also observed, especially in subjects with the highest insulin basal
354 values (> 50 μU/mL). These effects may be related to the processes described in other
355 mechanistic studies on the role of polyphenols in insulin metabolism. In particular, several
356 animal studies have reported that grape, other berries or cocoa (all with a polyphenol
357 profile very similar to that of grape pomace) are able to modulate insulin production and

358 degradation [7] as well as to affect insulin signaling. This latter effect may take place by
359 decreasing serine-phosphorylated levels of the insulin receptor substrate 1 and preventing
360 the inactivation of the glycogen synthase kinase 3/glycogen synthase pathway in the liver [27]
361 or by increasing the expression of fokhead box protein 1 (FOXO1) and peroxisome
362 proliferators-activated receptor gamma (PPAR γ) in muscle [13]. A recent study in subjects
363 with at least one component of MetS supplemented with a higher dose of grape pomace (20
364 g/day) reported a significant improvement in postprandial insulinaemia[24]. In contrast, a
365 recent clinical trial with a red wine polyphenol extract did not observe any improvement in
366 insulin sensitivity in obese subjects after an 8 weeks supplementation [28]. This discrepant
367 result may be due either to differences in the studied population or in the product provided.
368 A previous acute study with grape and pomegranate pomaces in subjects with abdominal
369 obesity showed a non-significant tendency towards the improvement of insulin sensitivity
370 when the product was provided 10 h before performing an OGTT (Pérez-Ramírez et al.,
371 *submitted*). It was suggested that this tendency could be due to the microbial-derived
372 polyphenol metabolites as main responsible of the biological effects of polyphenols [29],
373 comprising also non-extractable polyphenols [30]. Nevertheless, it is remarkable that this
374 potential effect of fruit pomaces on insulin regulation, observed after an acute intake, was
375 more clearly observed here after a chronic intake. This connects with the idea that
376 polyphenols exert subtle modifications that need long period to be observed in biochemical
377 risk parameters [31].
378 Leptin was also measured in samples from fasting time and 120 min in the OGTT.
379 Resistance to this hormone via impairment in its signaling or its transport towards the brain,
380 thus avoiding its anorexigenic effects, has been reported as an additional metabolic
381 alteration present in MetS [32]. Interestingly, studies in rats either with pure polyphenols or

382 polyphenol-rich extracts have shown that these compounds are able to counteract leptin
383 resistance [33]. The participants in this study exhibited a tendency towards leptin resistance,
384 with basal values about 25 ng/mL, concordant with their profile of overweight/obesity [34].
385 Slightly lower leptin values, although non-statistically significant, were observed 120 min
386 after the glucose overload; some studies have reported a significant decrease of leptin at
387 this time [35], while others did not find significant differences in this hormone during the
388 postprandial period suggesting other regulatory mechanisms for satiety [36]. Anyway, grape
389 pomace supplementation did not alter either fasting or postprandial leptin. Studies reporting
390 an effect of polyphenol supplementation on leptin levels in animal models indicate this
391 activity takes place by reducing adipose tissue and improving hypothalamic leptin signaling
392 [37]. Moreover, a decrease in leptin levels was reported in overweight subjects after
393 supplementation with a polyphenol-rich extract. Nevertheless, these studies were focused
394 on polyphenol classes different to those present in the grape pomace used here, what may
395 explain the lack of modifications in this marker.
396 No significant modifications were observed in all the other parameters evaluated
397 (cardiometabolic risk, anthropometry). This contrasts with previous studies reporting
398 beneficial effects on lipid profile in hypercholesterolemic subjects supplemented with a
399 product derived from grape pomace [23] or the ability of polyphenols to decrease uric acid
400 levels [38]. This discrepancy might be associated to the specific characteristics of subjects of
401 this study, which exhibited overweight/obesity. In this way, it was recently reported that
402 overweight subjects exhibited lower levels of phase II polyphenol metabolites, i.e. the
403 compounds responsible for their biological activities, after a repeated supplementation with
404 a grape seed extract, as compared with lean individuals [39]. Therefore, it may be

405 hypothesized that lower levels of circulating polyphenol metabolites in the present study
406 would decrease their potential biological effects.
407 Also, some of the discrepancies between this study and previous ones with grape products
408 may be derived from the fact that a specific approach of the present study was to use a
409 realistic dose (8 g/day, equivalent to less than 100 g fresh grapes/day), which could be
410 easily incorporated in a common diet. In contrast, other studies with grape extract or grape
411 pomace, reporting effects in parameters such as hypertension or fasting glucose used higher
412 doses (20g/d of grape pomace or 2 servings/d of grape) [24, 40] or less nutritional approaches
413 (6 pills/day) [41]. Indeed, a recent review concluded that a dose of 150-600 mg grape
414 products/kg body weight day was needed in order to obtain favorable effects on marker of
415 MetS [22]. This calculation applied to the present study yields a dose of 13-52 g/d in the
416 subjects participating in this study (mean weight, 89 kg). Nevertheless, this review did not
417 include any clinical trial with grape pomace, so there is still much research to be done
418 regarding this grape product with the specific composition described above.
419 Additionally, we determined several haematological and biochemical parameters related
420 with iron bioavailability, as it is widely known that polyphenolic compound can reduce iron
421 absorption [42], but there were no effects of the supplementation, which should be interpreted
422 considering the low dose of the supplement, the experimental period, and the characteristics
423 of the subjects who exhibited adequate iron stores (as determined by serum ferritin).
424 Finally, it should not be disregarded that, as a recent review highlighted [43], only a
425 combination of a polyphenol-rich diet including all the diversity of dietary polyphenols,
426 seems to be able to simultaneously counteract all the features present in MetS. Within this
427 context, the role shown here of grape pomace on the modulation of insulin would

contribute to the overall health-related properties of polyphenols, where the specific contribution of macromolecular antioxidants should also be considered.

CONCLUSIONS

A cross-over randomized clinical trial was performed in subjects with at least two factors of MetS. A 6-week supplementation with a realistic dose (8 g/day) of grape pomace, rich in both extractable and non-extractable polyphenols, significantly improved fasting insulineamia, without affecting other cardiometabolic risk parameters. A tendency towards an improvement in postprandial insulineamia was observed, particularly in those subjects with higher fasting insulin levels. These results show a promising role of grape pomace as coadjuvant for keeping standard insulin values, which should be further studied in subjects exhibiting hyperinsulineamia.

Acknowledgments

This study was funded by the Spanish Ministry of Science and Innovation (MINNECO-FEDER, grant: AGL2014-55102-JIN). A. G.-N. was funded by Youth Employment Initiative (YEI) from the European Social Fund (ESF). The authors are grateful to the Analysis Services Unit of ICTAN for microbiological, mineral, and chromatographic analyses. Dr. Laura Barrios, from the Statistics Department, Computing Center (SGAI-CSIC) is also thanked for her assistance with statistical treatment. Mr. Benjamín Brito-Fernández is thanked for technical assistance during the biochemical analyses. Roquesan Wineries are thanked for providing the raw materials of the dietary supplements. The authors are grateful to the volunteers for their participation in the study.

Conflict of interest

There are no conflicts to declare.

References

1. K. G. M. M. Alberti, R. H. Eckel, S. M. Grundy, P. Z. Zimmet, J. I. Cleeman, K. A. Donato, J. C. Fruchart, W. P. T. James, C. M. Loria and S. C. Smith. *Circul.*, 2009, **120**, 1640-1645.

2. A. Festa, R. D'agostino Jr, G. Howard, L. Mykkänen, R. P. Tracy and S. M. Haffner. *Circul.*, 2000, **102**, 42-47.

3. E. Hopps, D. Noto, G. Caimi and M. R. Averna. *Nutr. Metab. Cardiovasc. Dis.*, 2010, **2**0, 72-77.

4. A. Scuteri, S. Laurent, F. Cucca, J. Cockcroft, P. G. Cunha, L. R. Mañas, F. U. M. Raso, M. L. Muiesan, L. Ryliškyte, E. Rietzschel, J. Strait, C. Vlachopoulos, H. Völzke, E. G. Lakatta and P. M. Nilsson. *Eur. J. Prev. Cardiol.*, 2015, **22**, 486-491.

5. A. Barrett, T. Ndou, C. A. Hughey, C. Straut, A. Howell, Z. Dai and G. Kaletunc. *J. Agric. Food Chem.*, 2013, **61**, 1477-1486.

6. C. Bladé, L. Arola and M. J. Salvadó. *Molec. Nutr. Food Res.*, 2010, **54**, 37-59.

7. A. Castell-Auví, L. Cedó, V. Pallarès, M. T. Blay, M. Pinent, M. J. Motilva, S. Garcia-Vallvé, G. Pujadas, P. Maechler and A. Ardévol. *J. Nutr. Biochem.*, 2012, **23**, 1565-1572.

8. E. J. Chou, J. G. Keevil, S. Aeschlimann, D. A. Wiebe, J. D. Folts and J. H. Stein. *Am. J. Cardiol.*, 2001, **88**, 553-555.

9. C. G. Fraga, M. Galleano, S. V. Verstraeten and P. I. Oteiza. *Molec. Aspects. Med.*, 2010, **31**, 435-445.

10. S. Medjakovic and A. Jungbauer. *Food Function*, 2013, **4**, 19-39.

11. R. Zamora-Ros, C. Andres-Lacueva, R. M. Lamuela-Raventós, T. Berenguer, P. Jakszyn, A. Barricarte, E. Ardanaz, P. Amiano, M. Dorronsoro, N. Larrañaga, C. Martínez, M. J. SáNchez, C. Navarro, M. D. Chirlaque, M. J. Tormo, J. R. Quirós and C. A. González. *J. Am. Diet Assoc*. ,2010, **110**, 390-398.

12. R. Puupponen-Pinia, T. Seppänen-Laakso, M. Kankainen, J. Maukonen, R. Törrönen, M. Kolehmainen, T. Leppänen, E. Moilanen, L. Nohynek, A.M. Aura, K. Poutanen, F.A. Tomás-Barberán, J.C. Espín, K. M. Oskman-Caldentey. *Molec. Nutr. Food Res*., 2013, **57**, 2258-63.

13. E. Seo, S. Kim, S. J. Lee, B.C. Oh and H. S. Jun. *Nutrients*, 2015, **7**, 3038-53.

14. D. Klimis-Zacas, S. Vendrame, A.S. Kristo. *J. Berry Res*., 2016, **2**, 225-36.

15. J. Pérez-Jiménez, M. E. Díaz-Rubio and F. Saura-Calixto. *Nutr Res Rev*, 2013, **26**, 118-129.

16. I. Pérez-Ramírez, R. Reynoso-Camacho, F. Saura-Calixto and J. Pérez-Jiménez. *J. Agric. Food Chem.*, 2018, **24**, 661-73.

17. J. Woerdeman, E. Van Poelgeest, J. C. F. Ket, E. C. Eringa, E. H. Serné and Y. M. Smulders. *Eur. J. Clin. Nutr.*, 2017, **71**, 1381-92.

18. D. Kammerer, A. Claus, R. Carle and A. Schieber. *J. Agric. Food Chem.*, 2004, **52**, 4360-4367.

490 **19.** D. Grassi, G. Desideri, S. Necozione, C. Lippi, R. Casale, G. Properzi, J. B. Blumberg
491 and C. Ferri. *J. Nutr*., 2008, **138**, 1671-1676.

492 **20.** D. B. Allison, F. Paultre, C. Maggio, N. Mezzitis and F. X. Pi-Sunyer. *Diabetes Care*,
493 1995, **18**, 245-250.

494 **21.** J. Pérez-Jiménez and F. Saura-Calixto. *Nutr. Res. Rev.*, 2008, **21**, 158-173.

495 **22.** M. Akaberi and H. Hosseinzadeh. *Phytotherapy Res.*, 2016, **30**, 540-556.

496 **23.** J. Pérez-Jiménez, J. Serrano, M. Tabernero, S. Arranz, M. E. Díaz-Rubio, L. García-
497 Diz, I. Goñi and F. Saura-Calixto. *Nutr*, 2008, **24**, 646-653.

498 **24.** I. Urquiaga, S. D'acuña, D. Pérez, S. Dicenta, G. Echeverría, A. Rigotti and F.
499 Leighton. *Biol. Res.*, 2015, **48**, 49-58.

500 **25.** V. Kumar, A. K. Sinha, H. P. S. Makkar, G. De Boeck and K. Becker. *Cr. Rev. Food*
501 *Sci. Nutr.*, 2012, **52**, 899-935.

502 **26.** N. Motamed, S. J. H. Miresmail, B. Rabiee, H. Keyvani, B. Farahani, M. Maadi and F.
503 Zamani. *J. Diabetes Complications*, 2016, **30**, 269-274.

504 **27.** I. Cordero-Herrera, M. Martín, F. Escrivá, C. Álvarez, L. Goya and S. Ramos. *J. Nutr.*
505 *Biochem.*, 2015, **26**, 704-712.

506 **28.** J. Woerdeman, D. Del Rio, L. Calani, E. C. Eringa, Y. M. Smulders and E. H. Serné.
507 *Diab. Obesity Metabolism*, 2018, **20**, 206-210.

508 **29.** G. Williamson and M. N. Clifford. *Biochem. Pharmacol.*, 2017, **139**, 24-39.

509 **30.** A. González-Sarrías, J. C. Espín and F. A. Tomás-Barberán. *Trends Food Sci.*
510 *Technol.*, 2017, **69**, 281-88.

511 **31.** S. De Pascual-Teresa and M. N. Clifford. *J. Agric. Food Chem.*, 2017, **65**, 8093-8095.

512 **32.** S. B. Patel, G. P. Reams, R. M. Spear, R. H. Freeman and D. Villarreal. *Curr.*
513 *Hypertension Reports*, 2008, **10**, 131-137.

514 **33.** G. Aragonès, A. Ardid-Ruiz, M. Ibars, M. Suárez and C. Bladé. *Molec. Nutr. Food*
515 *Res.*, 2016, **60**, 1789-1803.

516 **34.** C. H. Jung and M. S. Kim. *Arch. Pharmacol. Res.*, 2013, **36**, 201-207.

517 **35.** M. P. Maziarz, S. Preisendanz, S. Juma, V. Imrhan, C. Prasad and P. Vijayagopal.
518 *Nutr. J.*, 2017, **16**, 14-23.

519 **36.** J. F. Carroll, K. A. Kaiser, S. F. Franks, C. Deere and J. L. Caffrey. *Obesity*, 2007, **15**,
520 2974-2983.

521 **37.** J. G. Franco, C. P. Dias-Rocha, T. P. Fernandes, L. Albuquerque Maia, P. C. Lisboa, E.
522 G. Moura, C. C. Pazos-Moura and I. H. Trevenzoli. *Eur. J. Nutr.*, 2016, **55**, 601-610.

523 **38.** A. Kerimi and G. Williamson. *Molec. Nutr. Food Res.*, 2016, **60**, 1770-1788.

524 **39.** J. A. Novotny, T. Y. Chen, A. I. Terekhov, S. K. Gebauer, D. J. Baer, L. Ho, G. M.
525 Pasinetti and M. G. Ferruzzi. *Molec. Nutr. Food Res.*, 2017, **61**, Article number 1700043.

526 **40.** J. Barona, J. C. Aristizabal, C. N. Blesso, J. S. Volek and M. L. Fernandez. *J. Nutr.*,
527 2012, **142**, 1626-1632.

528 **41.** R. Draijer, Y. De Graaf, M. Slettenaar, E. De Groot and C. I. Wright. *Nutrients*, 2015,
529 **7**, 3138-3153.

530 **42.** R. Blanco-Rojo and M. P. Vaquero. I*Innovative Food Sci. Emerging Technol.*, ***i****n press*,
531 DOI: 10.1016/j.ifset.2018.04.015

532 **43.** G. Chiva-Blanch and L. Badimon. *Oxid. Med. Cellular Longevity*, 2017, Article ID
533 5812401.

534

535

FIGURE CAPTIONS

Figure 1. CONSORT flow diagram trough the phases of a randomized, cross-over clinical trial on the effects of grape pomace in subjects at high cardiometabolic risk

Figure 2. Plasma leptin in subjects at high cardiometabolic risk before and after 6 weeks of daily supplementation with grape pomace, in fasting state (dark grey) and 120 min after an oral glucose overload (black) Data are represented as mean with their standard errors. No statistical differences between treatments (t-test for paired samples).

TABLES

Table 1. Metabolic syndrome risk factors among participants in the clinical trial with grape pomace besides overweight (55% of the subjects) or obesity (45% of the subjects).

Risk factor	Number of participants	
Blood pressure		32
Alone	5	
+ Glucose	11	
+ Glucose + HDL-cholesterol	7	
+ Glucose + triglycerides	2	
+ HDL-cholesterol	4	
+ Triglycerides	3	
Glucose		7
Alone	4	
+ HDL-cholesterol	2	
+ Triglycerides	1	
HDL-cholesterol alone		8
Triglycerides alone		1
Simultaneous five risk factors		1
Total		49

Cut-off values: Glucose ≥ 100 mg/dL; HDL-cholesterol ≤ 50 mg/dL female, ≤ 40 mg/dL male; triglycerides, ≥ 150 mg/dL; systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; BMI ≥ 25 kg/m^2.

Table 2. Plasma glucose and insulin during an Oral Glucose Tolerance Test in subjects at high cardiometabolic risk supplemented with grape pomace for 6 weeks

	Glucose				Insulin			
	0 weeks		6 weeks		0 weeks		6 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Concentration[1]								
0 min	98	2	100	2	8.5	0.8	5.5**	0.9
30 min	157	6	161	6	59.8	8.2	66.7	8.7
60 min	153	10	152	8	80.0	10.7	82.3	10.6
120 min	109	7	104	5	61.8	12.8	50.8	11.1
AUC								
0-30 min	3,926	136	3,955	120	1,062	139	1,095	139
0-60 min	7,616	318	7,655	270	2,687	338	2,623	335
0-120 min	12,471	443	12,388	373	4,247	816	3,416	721

[1] Glucose, mg/dL; insulin, μU/mL; AUC (glucose), mg/dL x min; AUC (insulin), μU/mL x min. ** Time effect, $P < 0.01$. Comparisons were performed using t-test for paired samples.

Table 3. Parameters of insulin sensitivity in subjects at high cardiometabolic risk supplemented with grape pomace for 6 weeks.

	Control period				Supplementation period			
	0 weeks		6 weeks		0 weeks		6 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
HOMA-IR	1.8	0.1	2.1	0.2	2.1	0.4	1.4**	0.3
QUICKI	0.35	0.004	0.35	0.004	0.35	0.007	0.42*	0.09

* $P < 0.05$; ** $P < 0.01$. Comparisons were performed using t-test for paired samples.
HOMA-IR, homeostatic model assessment- insulin resistance; QUICKI, quantitative insulin sensitivity check index

Table 4. Cardiometabolic markers in subjects at high cardiometabolic risk supplemented with grape pomace for 6 weeks.

	Control period				Supplementation period			
	0 weeks		6 weeks		0 weeks		6 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Lipid profile								
Total cholesterol (mg/dL)	202	28	195	26	201	29	190	27
HDL cholesterol (mg/dL)	50	13	49	7	47	7	48	7
LDL cholesterol (mg/dL)	124	17	118	15	121	17	112	16
Triglycerides (mg/dL)	143	20	137	21	147	21	150	21
Blood pressure								
Systolic (mmHg)	118	17	117	17	118	17	120	17
Diastolic (mmHg)	82	11	82	12	82	12	84	12
Others								
Plasma uric acid (mg/dL)	5.8	0.8	5.9	0.8	6.0	0.8	6.0	0.8
Urine uric acid (mg/g creatinine)	413	68	422	64	490	70	460	66
Fibrinogen (mg/dL)	351	47	318*	46	341	48	332*	47
High sensitive C reactive protein	0.3	0.07	0.4	0.07	0.3	0.07	0.4	0.07
AspAT (U/L)	21.9	3.1	20.1	3.0	22.6	3.2	21.4	3.1
ALT (U/L)	25.8	3.6	22.7	3.5	25.6	3.6	24.9	3.6

AspAT, asparte aminotransferase; ALT, alanine aminotransferase.* indicates time effect ($P < 0.05$)

Table 5. Hemogram and iron status in subjects at high cardiometabolic risk supplemented with grape pomace for 6 weeks.

	Control period				Supplementation period			
	0 weeks		6 weeks		0 weeks		6 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Hemogram								
Erythrocytes (10^6/μL)	5.0	0.1	4.9	0.1	5.0	0.1	4.9	0.1
Hemoglobin (g/dL)	15.0	0.2	14.9	0.2	15.0	0.2	14.8	0.2
Hematocrit (%)	45.4	0.5	44.7*	0.4	45.1	0.5	43.8*	0.5
MCV (fl)	90.7	0.7	91.0	0.7	90.0	0.7	90.1	0.7
MCH (pg)	30.0	0.3	30.4	0.3	29.8	0.3	30.5	0.3
MCHC (g/dL)	33.1	0.1	33.4*	0.1	33.1	0.1	33.9*	0.1
Erythrocytes distribution index (%)	13.4	0.1	12.9*	0.1	13.4	0.1	13.2*	0.1
Platelets (10^3/μL)	262	10	255	9	278	10	259	9
MPV (fl)	9.1	0.1	9.6*	0.1	8.9	0.1	9.0*	0.2
Iron status								
Iron (μmol/L)	17.3	0.9	15.9	0.8	16.0	0.8	15.0	0.7
Transferrin (g/L)	2.9	0.1	2.8	0.1	2.9	0.05	2.8	0.1
TIBC (μmol/L)	73.8	1.6	71.3	1.3	72.6	1.2	71.1	1.4
Transferrin saturation (%)	23.9	1.3	22.8	1.3	23.3	1.1	21.8	1.2
Soluble transferrin receptor (μg/mL)	0.9	0.04	0.9	0.05	1.0	0.05	1.1	0.05
Ferritin (ng/mL)	114.3	13.2	109.6	12.0	121.5	14.4	111.2	12.0

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume; TIBC, total iron-binding capacity.* indicates time effect ($P < 0.05$)

Table 6. Anthropometric measurements in subjects at high cardiometabolic risk supplemented with grape pomace for 6 weeks.

	Control period				Supplementation period			
	0 weeks		6 weeks		0 weeks		6 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
General parameters								
Body Mass Index (kg/m^2)	30.6	0.7	30.5	0.7	30.9	0.8	30.9	0.8
Abdominal perimeter (cm)	102.9	1.8	103.0	1.9	102.6	1.8	102.4	1.8
Waist-to-height ratio	0.6	0.01	0.6	0.01	0.6	0.01	0.6	0.01
Waist-to-hip ratio	0.9	0.01	0.9	0.01	0.9	0.01	0.9	0.01
Metabolic age	49.6	1.8	49.4	1.9	49.8	1.9	4.7	1.9
Visceral fat index	10.7	0.7	10.8	0.7	10.7	0.7	10.5	0.7
Whole body bioimpedance								
Fat (%)	32.8	1.3	32.3	1.3	32.7	1.3	32.5	1.4
Muscle (kg)	56.1	1.6	56.2	1.6	56.0	1.5	56.1	1.6
Minimum bone mass (kg)	3.0	0.1	3.0	0.1	3.0	0.08	3.0	0.08
Water (%)	48.7	0.8	49.0	0.8	48.7	0.8	48.9	0.99
Abdominal bioimpedance								
Fat (%)	32.9	1.2	32.5	1.2	32.6	1.2	32.3	1.2
Muscle (kg)	30.5	0.8	30.5	0.8	30.4	0.7	30.6	0.8

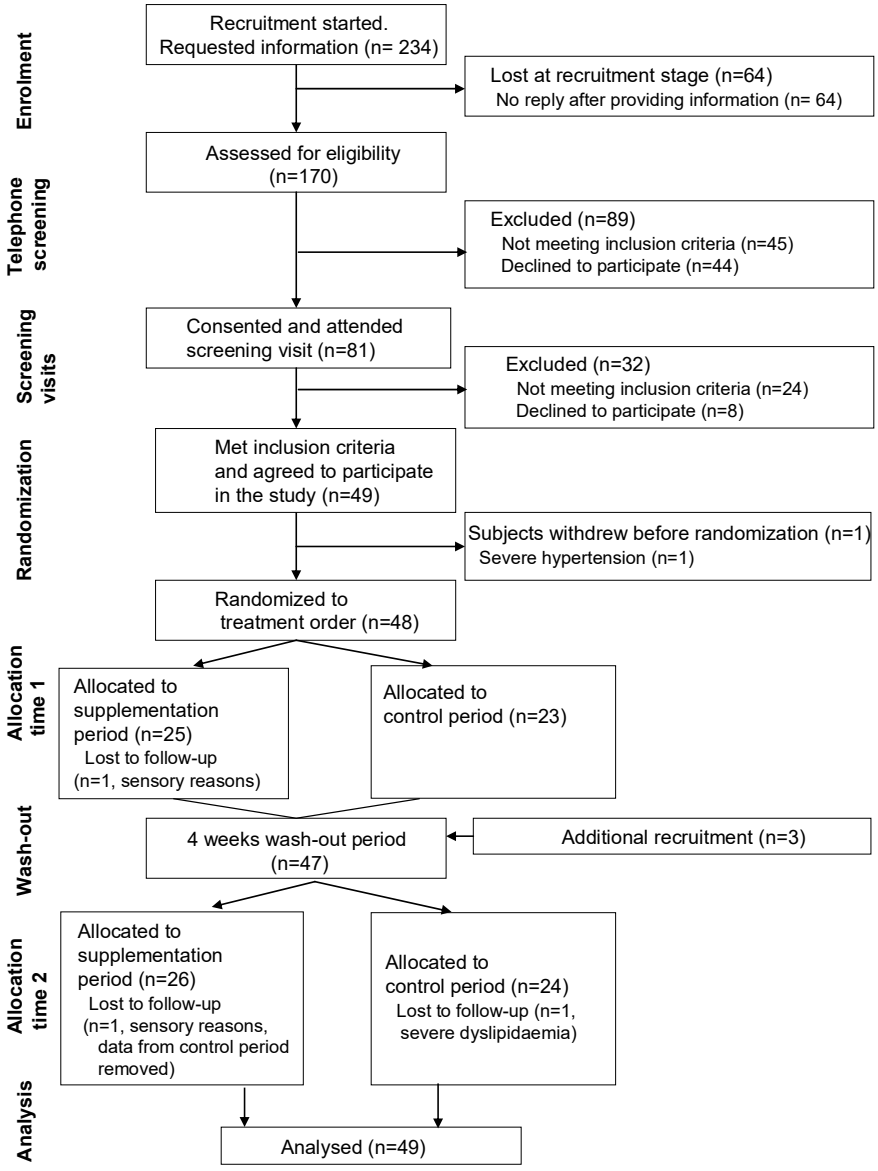
Enrolment
Recruitment started.
Requested information (n= 234)
Lost at recruitment stage (n=64)
No reply after providing information (n= 64)
Telephone screening
Assessed for eligibility
(n=170)
Excluded (n=89)
Not meeting inclusion criteria (n=45)
Declined to participate (n=44)
Screening visits
Consented and attended
screening visit (n=81)
Excluded (n=32)
Not meeting inclusion criteria (n=24)
Declined to participate (n=8)
Randomization
Met inclusion criteria
and agreed to participate
in the study (n=49)
Subjects withdrew before randomization (n=1)
Severe hypertension (n=1)
Randomized to
treatment order (n=48)
Allocation time 1
Allocated to
supplementation
period (n=25)
Lost to follow-up
(n=1, sensory reasons)
Allocated to
control period (n=23)
Wash-out
4 weeks wash-out period
(n=47)
Additional recruitment (n=3)
Allocation time 2
Allocated to
supplementation
period (n=26)
Lost to follow-up
(n=1, sensory reasons,
data from control period
removed)
Allocated to
control period (n=24)
Lost to follow-up (n=1,
severe dyslipidaemia)
Analysis
Analysed (n=49)

Figure 1.

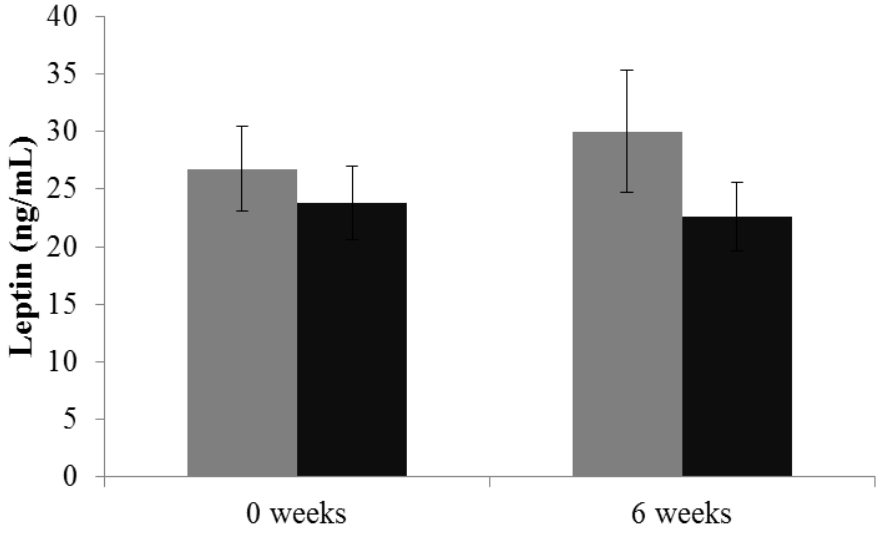

Figure 2.